Genetic Transformation using Ri Plasmid and Establishment of Cell Suspension Cultures of the Medicinally Important Plant *Piper longum* L.

A synopsis of the proposed work for
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Submitted by

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INTRODUCTION

Medicinal plants constitute an important group of non-wood forest products. Approximately 80% of the world population uses plants as a source of medicine for healthcare (WHO, 2008). Out of the total 4,22,000 flowering plants reported from the world, more than 50,000 are used for medicinal purposes (Govaerts, 2001). WHO has estimated the present demand for medicinal plants is approximately US $14 billion per year. The demand for medicinal plant-based raw materials is growing at the rate of 15 to 25% annually and is likely to increase to more than US $5 trillion in 2050 (Sharma, 2004; Anees *et al.*, 2009).

India is one of the 12 biodiversity centres of the world with over 45,000 different plant species. The climatic diversity of India is unmatched as she has 16 different agro-climatic zones, 10 vegetative zones, 25 biotic provinces and 426 biomes and is one of 12 biodiversity centres of the world. Over 45,000 plant species have been recorded from India of which about 15,000-20,000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional systems (Joy *et al.*, 1998; Shiva, 1996).

There are more than 7800 manufacturing units involved in the production of natural health products and traditional plant based formulations in the country producing around 25,000 effective plant-based formulations. An estimated 2000 tones of medicinal plants as raw material are required annually (Anees *et al.*, 2009) generating approximately US $1 billion per annum (Warude *et al.*, 2004*;* Kala *et al.*, 2006).

Collection from wild which is the traditional practice is not enough to meet the demand, therefore, large scale cultivation is needed. There is worldwide realization that unless the basic information on the distribution, diversity and survival strategies of medicinal plants is available, their conservation and sustainable utilization may not be possible. Several medicinal plants that are being over-exploited are now growing scattered in their native habitats. Due to environmental degradation, there has also been a decoupling of plant-pollinator relationship and many of the endangered medicinal plants are facing reproductive stress.

Commercial cultivation of medicinal plants would require quality planting material on a large scale. In order to generate quality planting material, it is essential to know the existing variation in the natural population of the medicinally important plants and develop in vitro methods for large-scale production and conservation of these species.

The importance of medicinal plants is because of the secondary metabolites these plants possess. The secondary metabolites are extracted and used either as crude or further partitioned to isolate the active principle(s). Since the yield of the crude or the active principle(s) is in milligram quantities, the extraction requires to be initiated from very large quantities of dry plant materials. Plant cell cultures and hairy root cultures are being used extensively for in vitro production of several secondary metabolites (Bhojwani and Dantu, 2013; Dantu and Bhojwani, 2005). In several instances, the in vitro production of useful secondary metabolites has been increased several fold by manipulating the culture and media conditions. In vitro production of rosmarinic acid from *Coleus blumei* could be increased nine-fold, while that of azadirectin from *Azadirecta indica* was increased 150-fold (Cai, 2012; Srivastava and Srivastava, 2007).

Hairy root cultures produced through Ri plasmid transfer by *Agrobacterium rhizogenes* is an important in vitro method for production of important and useful secondary metabolites. Varying culture conditions and media constituents yield of target products by hairy root cultures has been improved several folds (Bhojwani and Dantu, 2013). Growth rates as high as 2,500-5,000 fold increase of root biomass could be obtained in the hairy root cultures of *Hyocyamus muticus* (Flores *et al.,* 1987). Production of secondary metabolites by hairy root cultures was equal or greater than in vivo roots of *Beta vulgaris, Valeriana officinalis* and *Lupinus.* Both suspension cell cultures and hairy root cultures with appropriate modifications could be scaled up for production of target compounds in bioreactors (Bhojwani and Dantu, 2013).

The in vitro strategy for production of secondary metabolites and the target compounds is the green path for fulfilling the needs of the natural product based pharmaceutical industries. These strategies will amply take care of sustainable utilization of the medicinal plants.

OBJECTIVES OF THE PRESENT STUDY

For the present study, an important medicinal plant *Piper longum* (common name Pippali or Piplamul or Long Pepper; family Piperaceae) has been selected. Long pepper is a slender aromatic climber with alternate leaves that are ovate, acute to acuminate apex, entire margin and glabrous (Viswanathan, 1995). Inflorescence is a spike with unisexual small achlamydeous densely packed flowers and form very close clusters of small greyish green or darker grey berries. The plant is a native of Indo-Malaya region and grows wild in Assam, West Bengal, Uttar Pradesh, Madhya Pradesh, Maharashtra, Kerala, Karnataka and Tamil Nadu (Joy *et al.*, 1998). The spikes are rich in piperine and piperidine which gives the ripe fruits the characteristic pungent taste. The spike and root are widely used in ayurvedic and unani systems of medicine particularly for diseases of respiratory tract.

Piper longum is rarely cultivated. It is still extensively collected from the wild, threatening the very existence of the plant (Nair, 2000). Effective micropropagation protocol has been developed in our laboratory (Rani and Dantu, 2012). Further research is being carried out in the lab to understand the spatio-temporal basis of in vitro shoot differentiation in this plant.

Till far no attempt has been made to produce secondary metabolites in tissue cultures of *P. longum*. The present work envisages to establish 'hairy root' cultures through *Agrobacterium rhizogenes* infection and callus suspension cultures for possible in vitro secondary metabolite production in this plant with the following specific objectives:

1. To establish cell suspension cultures from a suitable explant on an appropriate medium.
2. To transform plants with Ri plasmid from *Agrobacterium rhizogenes* to generate and establish 'Hairy Root' cultures.
3. Analysis for secondary metabolites produced in vitro in the cell suspensions and 'hairy roots'.

REVIEW OF LITERATURE

Piper longum is the third most economically important species of the genus *Piper* after *P. nigrum* and *P. betle*. It is rich in two major alkaloids, piperine and piperidine. In traditional and Ayurvedic medicines, mature spikes of the female plants (long pepper), thick stems, roots and leaves are used against bronchial diseases, dyspepsia, worms and amoebiosis. The spikes have also been effectively used as an anti-inflammatory, memory enhancing and aphrodisiac agent. When used in conjunction with a drug piperine has been shown to improve the drug's bioavailability. *P. longum* has also gained significance in processing of essential oils and oleoresins that are used as food additives, in pharmaceuticals and as insecticide (CSIR, 1989; Rani and Dantu, 2012).

The cultivation of *Piper longum* is rare. Due to continuous demand and extensive collection the plant is threatened in the wild. There is an urgent need to cultivate and develop conservation strategies for it. As the tissue culture protocol for mass proliferation and conservation offers a viable alternative, many regeneration protocols for this plant have been reported earlier from shoot tips, root, leaf, node, internode, and petiole (Rani and Dantu, 2012).

Efficient and rapid tissue culture systems were developed for *Piper longum* through shoot tip multiplication and direct regeneration. De novo shoot regeneration from callus cultures through organogenesis (Bhat *et al.,* 1992) or direct shoot regeneration from leaf bases (Soniya and Das, 2002) or through axillary bud break (Parida and Dhal, 2011) have been reported in *P. longum*. A single-step, high-frequency regeneration protocol has been standardized for *Phytophthora*-resistant wild pepper, *Piper colubrinum* using root, internode, leaf and petiole explants derived from in vitro plantlets. The elongated shoots were rooted directly in soil and hardened in the greenhouse (Kelkar *et al.,* 1998). Direct somatic embryogenesis from in vitro cultured leaf segments was also reported in *Piper colubrinum* (Yusuf *et al.,* 2001). Morphogenetic potential of root, leaf, node and internode explants of three cultivated *Piper* species was investigated to develop a reliable plant regeneration protocol. *P. longum* was the most responsive followed by *P. betle* and *P. nigrum*. (Bhat *et al.,* 1995).

In a recent study, three explants, namely, nodal, internodal and petiolar segments were used to establish in vitro cultures of *Piper longum*. Multiple shoots were induced on semi-solid MS medium supplemented with 1 mg/L 6-benzyladenine (BA). Addition of ascorbic acid (40 mg/L) considerably reduced browning of tissue and medium. Best shoot regeneration was observed from petiolar explants and was, therefore, used for all further studies. An indexing method was introduced for checking bacterial contamination in well established shoot multiplication cultures. It was found that bacterial infection was quite high in shoots derived from nodal and internodal explants while it was least in those obtained from petiolar segments. Only shoots that indexed negative for endogenous bacteria were used for proliferation and in vitro conservation studies. A conservation strategy was also developed. The shoot cultures could be maintained without subculturing for as long as eight weeks in MS medium supplemented with 1 mg/L paclobutrazol (PBZ) and 40 mg/L ascorbic acid (Rani and Dantu, 2012).

The fruits contain 1.0-2.5% volatile oil and 5-9% alkaloids rich in piperine, chavicine, piperidine, piperetine and a resin (Sawangjaroen *et al.,* 2004). The ripe spikes have pungent pepper-like taste and stimulate salivation and causes numbness of the mouth. Pharmacological and clinical studies have revealed that piperine has CNS depressant, antipyretic, analgesic, anti-inflammatory (Lee *et al.,* 1984), antioxidant (Khajuria *et al.,* 1994), and hepatoprotective (Kaul and Kapil, 1993) activities. Piperine has also been shown to enhance the bioavailability of several drugs such as sulfadiazine, tetracycline, streptomycin (Atal *et al.,* 1980; Kanaki *et al.,* 2008), rifampicin, pyrazinamide, isoniazid, ethambutol (Zutshi *et al.,* 1985), and phenytoin (Bano *et al.,* 1987). The alcoholic extract of the fruits of *Piper longum* and its component piperine demonstrated immunomodulatory and antitumor activity. The extract and piperine was found to be 100% toxic to Dalton's lymphoma ascites (DLA) cells and Ehrlich ascites carcinoma (EAC) cells (Sunila and Kuttan, 2004). A methanolic extract of its fruit powder was found to be effective against mosquito larvae, whereas an ethanolic extract had an anti-amoebic activity (Prasad *et al.,* 2005).

Plant cell suspension cultures are widely used as a convenient tool for the investigation of a wide range of phenomena bypassing the structural complexity and

control of the plant system. The homogeneity of an in vitro cell population, large availability of study material, high rate of cell growth and good reproducibility of conditions make cell suspensions suitable for the analysis of complex physiological processes at the cellular and molecular levels. Moreover, plant cell cultures provide a valuable platform for the production of high-value secondary metabolites and other substances of commercial interest (Mulabagal and Tsay, 2004; Moscateiallo *et al.*, 2013). Further, it is easy to scale up suspension cell cultures to bioreactor levels for large scale production of target compounds (Bhojwani and Dantu, 2013).

Plant cell culture is the only economically feasible way of producing some high-value metabolites (e.g., paclitaxel) from rare and/or threatened plants (Milen *et al.*, 2009). Plant cell culture has been explored in the production of numerous terpenoid and terpenoid-derived compounds, most notably high-value pharmaceutical products such as the TIAs from *Catharanthus roseus*, taxanes from *Taxus sp*. and artemisinin from *Artemisia annua*. Cell culture has also been investigated for production of other pharmaceutically active agents such as shikonin from *Lithospermum erythrorhizon*, berberine from *Coptis japonica*, camptothecin from both *Camptotheca acuminate* and *Nothapodytes foetida*, and hypericins from *Hypericum perforatum*. Many processes have been developed using plant suspension cultures as a result of the ease in scale up through modified traditional optimization strategies developed for microbial and mammalian cell systems (Bhojwani and Dantu, 2013).

Taxus sp. cell suspension culture represents a success of plant cell culture technology in the United States with its proven feasibility by Bristol-Myers Squibb and Phyton Biotech, Inc. (a DFP Pharmaceuticals company) in supplying paclitaxel for Bristol-Myers Squibb's Taxol formulation. In addition, Samyang Genex (Korea) has commercialized a process for the production of paclitaxel via plant cell culture. Plant cell culture is not only useful in the supply of valuable terpenoids, but can also be extremely valuable in isolating previously unidentified terpenoid metabolic genes. The best example is elucidation of the paclitaxel biosynthetic pathway (Roberts, 2007).

In most plant cells, the production of secondary metabolites is not associated with cell growth. Secondary metabolites are often produced under stress conditions or at the

end of the growth cycle, i.e. during the stationary phase. It has been observed that cells in suspension cultures often synthesize very small amounts of secondary metabolites. Various strategies, such as the selection of high-yielding cell lines, manipulation of media, culture conditions, immobilization, and elicitation have been employed to improve the production of secondary metabolites from plant cell culture for their industrial exploitation (Bhojwani and Dantu, 2013; Malik *et al.,* 2013).

Another approach for the production of secondary metabolites is 'Hairy Root Cultures' (HRCs). Roots transformed with *Agrobacterium rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions. A number of plant species including many medicinal plants have been successfully transformed with *Agrobacterium*. Transformed root cultures are fast-growing and genetically stable which can also be successfully cultured in large-scale bioreactor. Transformed roots are able to produce secondary metabolites at levels that are often comparable to or greater than that of the intact plants (Sevon and Caldentey, 2002; Bhojwani and Dantu, 2013).

Azadirachtin, a well-known biopesticide is a secondary metabolite conventionally extracted from the seeds of *Azadirachta indica*. Liquid cultures of hairy roots from this plant were established in shake flask to study the kinetics of growth and azadirachtin production. A biomass production of 13.3 g/L dry weight was obtained after 25 days of cultivation period with an azadirachtin yield of 3.3 mg/g root biomass (Srivastava and Srivastava, 2012).

An efficient transformation system for the medicinal and aromatic plant, *Pogostemon cablin* was developed by using agropine-type *Agrobacterium rhizogenes*. The results presented provide a solid foundation for production of patchouli essential oil from hairy roots or its regenerated plants and also provide possibilities for utilization of artificial polyploidization or chemical mutation of hairy roots for improving germplasm and breeding of a new cultivar of *P. cablin* (Shi *et al.,* 2011).

Transformed roots of *Tylophora indica* produced shoots or embryogenic calli in the absence of hormone treatments though hormonal treatment was required to regenerate

shoots in root explants of wild type control plants. Transformed plants showed morphological features typically seen in transgenic plants produced by *A. rhizogenes*. Plants regenerated from transformed roots showed increased biomass accumulation (350-510% in the roots and 200-320% in the whole plants) and augmented tylophorine content (20-60%) in the shoots, resulting in a 160-280% increase in tylophorine production in different clones grown in vitro (Chaudhari *et al*., 2006).

Hairy roots of *Solenostemon scutellarioides* were induced by inoculation of leaf explants with *Agrobacterium rhizogenes*. Strain of the bacterium used greatly influenced the degree of hairy root formed. Acetosyringone was found to be useful for the enhancement of hairy roots formation (Saleh and Thuc, 2009). The transgenic hairy root lines of *Lithospermum canescens* have shown the enhanced production of antitumour naphthoquinones. An eight-fold increase in acetylshikonin and isobutyrylshikonin accumulation was achieved when 32-days old transransgenic roots of Lc1D line were transferred from LS to M9 medium for the subsequent 3 weeks of culture (Katarzyna *et al.,* 2012).

Hairy root cultures of *Atropa belladonna* were established by transformation with *A. rhizogenes* and showed that a rise in KNO_3 concentration causes a decline in hairy roots growth, and have a remarkable effect on the alkaloid content. The alkaloid concentrations obtained in the hairy roots were 3-20 times higher than that in the plants at 35 mM of KNO_3. Increasing the nitrate concentration in the medium of hairy roots also improved the hyoscyamine/scopolamine ratio, while it increased the scopolamine/hyoscyamine ratio in the studied plants (Chashmi *et al.,* 2010). In *Stephania suberosa*, effects of sucrose content, tyrosine, and medium strength on growth and dicentrine production of hairy root cultures were investigated. Sucrose at 6% (w/v) was an optimum content for the growth and dicentrine accumulation in *S. suberosa* hairy roots. The use of a precursor such as tyrosine enhanced the dicentrine production. The medium with 1.0 mM of tyrosine had the highest effect on dicentrine accumulation in hairy roots at day 40 of culture (Putalun *et al.,* 2009). In *Echium rauwolfii* the transformed hairy root cultures were exposed to different elicitors, such as methyl jasmonate (MJ), quercetin and salicylic acid in order to increase their productivity. MJ at

a concentration of 100 μM induced the accumulation of total alkaloids about 19-fold compared to the untreated control. The flavonoid quercetin at a concentration of 50 μM enhanced the pyrrolizidine accumulation approximately 6-fold (Abd El-Mawla, 2010).

PROPOSED METHODOLOGY

1. To establish cell suspension cultures of *Piper longum*
    - Establishment of axenic cultures from various explants
    - Selection of suitable explants for callus initiation and callus formation.
    - Initiation of cell suspension from callus.
    - Maintenance of cell suspensions.
    - Variation of culture conditions both nutritional and hormonal to promote in vitro secondary metabolite production.
    - Analysis of suspension cultures for secondary metabolites.
    - Standardization of media constituents and culture conditions for maximizing in vitro secondary metabolite production.

2. To transform *P. longum* plant with Ri plasmid from *Agrobacterium rhizogenes*
    - Selection of an efficient strain of *Agrobacterium rhizogenes* for hairy root formation.
    - Standardization of protocol of hairy root formation in *Piper longum.*
    - Mass production of hairy roots.
    - Analysis for integration of Ri plasmid in the *P. longum* genome.
    - Analysis of transformed roots for secondary metabolites.

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